# Mechanisms of Gal$\alpha$1-3Gal$\beta$1-4GlcNAc-R ($\alpha$Gal) expression on porcine valve endothelial cells

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**Objective:** We have previously reported that porcine valve endothelium does not express immunodetectable levels of the carbohydrate Gal$\alpha$1-3Gal$\beta$1-4GlcNAc-R (known as *$\alpha$Gal*), suggesting that fresh porcine valve may be immunoprivileged. In this study, we further investigated the mechanisms of $\alpha$Gal expression on porcine valve endothelial cells.

**Methods:** Primary cultures of porcine valvular endothelial cells were established and compared with porcine aortic endothelial cells and human vein endothelial cells. Immunoblotting, reverse transcriptase-polymerase chain reaction, and flow cytometry were used to compare the expression of $\alpha$Gal at both the protein and messenger RNA levels.

**Results:** Porcine valvular endothelial cells grew rapidly on a gelatin substrate. Similar to our previous in vivo results, valve endothelial cells expressed $\alpha$Gal much less intensely than did aortic endothelial cells. Porcine aortic endothelial cells expressed an isolectin B4 (isolectin B4 lectin *Bandeiraea simplicifolia*) immunodetectable band at 135 kd that was not visible on porcine valve endothelial cells or on human vein endothelial cells. Reverse transcriptase-polymerase chain reaction documented three transcripts of the $\alpha$Gal gene that were identically expressed on porcine valve and aortic endothelial cells. Furthermore, flow cytometry showed an almost identical surface profile between porcine aortic and valve endothelial cells, in contrast with human vein endothelial cells.

**Conclusions:** Cultures of primary valve endothelial cells were established and exhibited similar phenotypic patterns in vitro to those we have previously documented in vivo. RNA and flow cytometric analyses documented no difference between the RNA expression and surface protein profile for $\alpha$Gal, although whole-cell extracts demonstrated an immunodetectable band on Western blotting that was present on aortic endothelial cells but not on valve endothelial cells. These findings clarify the mechanism of expression of $\alpha$1,3galactosyltransferase gene expression in valve endothelial cells, suggesting that delayed rejection of fresh porcine cardiac valves may occur.

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Valve research during the past 4 decades has attempted to produce the ideal replacement valve. Current research has been broadly divided along the lines of mechanical versus bioprosthetic valves. Although the ideal prosthetic valve does not yet exist, its characteristics have been well defined.[1] It should provide excellent hemodynamic properties, provide durability, and have a minimal need for anticoagulation.

The development of a long-term viable bioprosthesis has been hampered by structural deterioration when placed into younger patients needing valve replacement.[2] It has been theorized that a viable bioprosthesis would contain regenerating fibroblasts and valve endothelial cells to provide a turnover of matrix and anti-

thrombotic factors. Fibroblasts could produce collagen to stiffen the underlying valve matrix. Valvular endothelial cells could provide the proper balance between procoagulant and anticoagulant forces to minimize the need for long-term anticoagulation. The hemodynamic benefits of bioprostheses might be realized without the long-term structural deterioration that some have linked to glutaraldehyde fixation. Concern has been expressed that a viable endothelial surface displays immunologically xenoreactive epitopes, such as major histocompatibility complex class II, intracellular adhesion molecule 1, CD54, and E-selectin, on the surface of valve endothelium.[3,4] For example, some centers have demonstrated that ABO mismatch is an independent predictor of graft deterioration, and typing thus may improve the long-term outcomes of homograft valves,[5] although this is controversial.[6]

The interaction between primate xenoreactive antibodies and the carbohydrate Gal$\alpha$1-3Gal$\beta$1-4GlcNAc-R ($\alpha$Gal) is the major acute obstacle faced when xenotransplanting porcine tissue.[7] The $\alpha$Gal epitope is synthesized by the glycosylation enzyme $\alpha$1,3galactosyltransferase. The $\alpha$Gal epitope is homologous to the ABO blood group antigens; in effect, pigs have an additional blood group antigen.

We have demonstrated that native porcine cardiac valves do not have any immunohistochemically detectable $\alpha$Gal on the surface, as shown by immunostaining with isolectin B4 (IB4) lectin.[8] Furthermore, when hearts from transgenic swine expressing human complement regulatory proteins (CD59 and decay-accelerating factor) are implanted into baboons, the valves, unlike the myocardium, are spared from immunoglobulin M and membrane attack complex deposition, thought to be initiated by the expression and binding of xenoreactive antigens to endothelially expressed $\alpha$Gal.[9] We later found that freshly harvested human cardiac valves have no immunohistochemically detectable ABO blood group antigens, which are the human homologs of $\alpha$Gal, on their surface.[10] We then attempted to use cell culture to define the surface epitopes on human vein endothelial cells and human saphenous vein endothelial cells. In culture, they appeared to lack ABH reactivity,[6,11] although they have been shown to stimulate human T-cell proliferation and interleukin 2 release.[3]

Because saphenous vein endothelial cells may not be a good proxy for valve endothelial cells, we established cultures of porcine aortic endothelial cells and porcine valve endothelial cells and applied Western blotting, immunohistochemical methods, RNA analysis, and flow cytometry to aortic and valve endothelial cells to investigate further the mechanism of $\alpha$Gal expression.

## Material and Methods

### Supplies

*Bandeiraea simplicifolia* IB4 lectin; both fluorescein isothiocyanate-conjugated and biotinylated IB4 lectin; *Ricinus communis* agglutinin I lectin (RCA lectin, or $\beta$Gal); *Thermophilus aquaticus (Taq)* DNA polymerase; gelatin (2% solution type B from bovine skin); mineral oil; heparin sulfate; protease inhibitor cocktail (pepstatin A, leupeptin, bestatin, aprotinin, and E-64; P 8340); bicinchoninic acid protein assay kit, collagenase (from *Clostridium histolyticum*); monoclonal anti-smooth muscle cell $\alpha$-actin (A-2547); diaminobenzidine; penicillin G, streptomycin, and glutamine solutions, trypsin-EDTA solution for endothelial cells; and Tri-Reagent were purchased from Sigma (St Louis, Mo). Oligonucleotide primers for human $\beta$-actin were purchased from Stratagene (LaJolla, Calif). Oligonucleotides created for reverse transcriptase-polymerase chain reaction (PCR) for $\alpha$Gal were purchased from Sigma Genosys (Woodlands, Tex). Precast agarose gels (4%), prestained molecular weight markers, and precut 0.20-$\mu$m polyvinylidene difluoride membranes were purchased from Invitrogen Corporation (Carlsbad, Calif). Casein (I-block) was purchased from Tropix (Bedford, Mass). Endothelial cell growth factor was purchased from (Roche Diagnostics Corporation, Laboratory Systems (Indianapolis, Ind). Human vein endothelial cells (catalog CRL-1730) and Kaighn F-12 medium were purchased from American Type Culture Collection (Manassas, Va). Primary cultures of porcine aortic endothelial cells were ordered from Cell Systems (Kirkland, Wash). The Vectastain Elite avidin-biotin complex kit was purchased from Vector Laboratories, Inc (Burlingame, Calif). Superscript II reverse transcriptase, medium 199 with Earle salts, Dulbecco phosphate-buffered saline solution lacking calcium or magnesium, and gentamicin were purchased from Life Technologies, Inc (Rockville, Md). Fetal calf serum (heat inactivated) was purchased from HyClone Laboratories Inc (Logan, Utah). Adult 50-kg female pigs were purchased from Parson Farms (Hadley, Mass). Sodium pentobarbital was purchased from Delmarva Laboratories (Midlothian, Va). Six-well tissue culture plates were purchased from Denville Scientific (Metuchen, NJ). Crystal/Mount was purchased from Biomeda Corp (Foster City, Calif).

### Immunohistochemical Studies

Cells were grown to near confluence on plastic tissue culture-treated slides. Cells were fixed for 10 minutes in ice-cold 10% methanol and phosphate-buffered saline solution. For lectins, a 1:100 solution of biotinylated IB4 lectin or RCA lectin was applied for 1 hour at room temperature. Slides were then developed by incubating for 30 minutes in avidin-biotin complex, which was then developed with diaminobenzidine. Washes for lectins were done with phosphate-buffered saline washes without polysorbate (Tween) 20. The high stringency of Tween 20 resulted in no staining (data not shown). Counterstaining was with Gill hematoxylin for 1 minute. Slides were then mounted with an aqueous mount. For smooth muscle cell $\alpha$-actin immunostaining, primary antibody was used at a concentration of 1:10,000 in phosphate-buffered saline solution and 1% bovine serum albumin for 1 hour. Washes were all performed in phosphate-buffered saline solution with Tween (0.5%). Secondary antibody incubation (1:2000 in phosphate-buffered saline solution with Tween) was for 1 hour at room temperature. Avidin-biotin complex reagent was then applied for 1 hour, and diaminobenzidine was used as a substrate for the horseradish peroxidase reaction.

Sections of tissue were used as a control for antibodies. Sections were cut 4-$\mu$m thick on a Leica microtome (Leica Microsystems Inc, Deerfield, Ill) from formalin-fixed, paraffin-embedded tissues. Standard deparaffinization and dehydration were performed. Tissue was treated with protease K for 10 minutes. Incubation in avidin-biotin complex reagent alone served as a nonspecific negative control preparation.

## Cell Culture: Porcine Valvular Endothelial Cells

Primary cultures of porcine valvular endothelial cells were prepared with the following modifications of previously published methods. Hearts were removed under aseptic conditions from domestic adult swine (50 kg) that were killed by injection of 87 mg/kg sodium pentobarbital. All animals were treated humanely in compliance with the Guide for the *Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996. The valve leaflets were removed from the aortic and mitral valves and placed into phosphate-buffered saline solution supplemented with antibiotics (100 U/mL penicillin G and 20 $\mu$g/mL gentamicin). A margin of leaflet was left attached to the anulus. Valve leaflets were washed free of red blood cells by three washes with phosphate-buffered saline and antibiotic solution. The valve leaflets were then placed in a 0.6-mg/mL collagenase solution (from *C hystoliticum*) in phosphate-buffered saline solution supplemented with 100 U/mL penicillin G and 20 $\mu$g/mL gentamicin at 37°C for 15 minutes. The valve leaflet was then gently shaken for 1 minute in medium 199. Cells that became dislodged were then placed by aliquots into a 96-well plate into which complete medium was added. Medium was changed every 48 hours. Cells were checked for expansion every day. Cells with fibroblastic or smooth muscle cell morphologic and growth characteristics, as evinced by spindle morphologic characteristics or lack of contact inhibition, were discarded. Cells of typical endothelial morphologic type were then expanded and confirmed to be of endothelial cell origin by immunohistochemical methods. These cells were used for subsequent experiments. Complete medium consisted of medium 199 with Earle salts supplemented with 20% heat-inactivated fetal calf serum, penicillin at 100 U/mL, streptomycin at 0.1 mg/mL, L-glutamine at 2 mmol/L, endothelial cell growth factor at 100 $\mu$g/mL, and heparin at 50 $\mu$g/mL. Cells were subcultured at near confluence with a standard trypsin (500 BAEE) and EDTA (180 $\mu$g/mL) detachment procedure.

Porcine aortic endothelial cells were obtained during first passage (<6 cumulative population doublings) and routinely used for experiments between passages 4 through 6. They were isolated from the aortas of large white pigs, as described by Ryan and Maxwell.[12] They were grown in complete medium 199, which was changed every 48 to 72 hours. Human vein endothelial cells were grown in Kaighn's F12 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/mL), streptomycin (0.1 mg/mL), and L-glutamine (2 mmol/L), endothelial cell growth factor (100 $\mu$g/mL), and heparin (50 $\mu$g/mL). Cells were subcultured near confluence with trypsin.

## Reverse Transcriptase-Polymerase Chain Reaction

RNA was extracted with previously described modifications[13] of the method of Chomczynski and Sacchi.[14] In brief, endothelial cells were lysed with 4 mL Tri-Reagent per 10-cm petri dish of confluent cells. Chloroform was added at one fifth final volume and the solution was vigorously vortex mixed. Nucleoprotein complexes were then allowed to dissolve by letting the homogenate sit for 15 minutes on ice. The solution was centrifuged for 30 minutes at 4°C and 12000*g*. The aqueous layer was then removed and mixed with an equal volume of isopropanol. The solution was precipitated overnight at −20°C. Subsequently, the isopropanol and RNA mixture was centrifuged at 12000*g* for 30 minutes at 4°C. The resulting RNA pellet was washed with a total of 6 mL of 70% ethanol and centrifuged again at 7500*g* at 4°C for 15 minutes. The RNA pellet extracted from cell culture was dissolved in 40 $\mu$L of water. RNA was quantified by absorbence at 260 nm, with a 260 nm/280 nm ratio of 1.7 to 1.9. All experiments were performed in triplicate with identical results, and a single representative autoradiograph is shown.

Reverse transcription was performed according to the Superscript II directions. In brief, 2 $\mu$g of total RNA was mixed with oligodeoxythymidine primer (0.5 $\mu$g/$\mu$L) and annealed for 10 minutes at 70°C and then placed on ice. PCR buffer (20-mmol/L tris[hydroxymethyl]aminomethane [Tris, pH 8.4] and 50-mmol/L potassium chloride), magnesium chloride (2.5 mmol/L), deoxyribonucleoside triphosphate (1 mmol/L), and dithiothreitol (10 $\mu$mol/L) were added. Samples were heated to 42°C for 5 minutes, at which point reverse transcriptase was added to the mixture. The reaction was allowed to proceed for 50 minutes and then terminated by 5 minutes of heating to 94°C. Five percent (1 $\mu$L) of this reaction was routinely used for PCR amplification.

The human $\beta$-actin primer consisted of the sense sequence 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′ and the antisense sequence 5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′, resulting in a 661-base pair (bp) fragment.[15] Reverse transcription of the RNA extracts was performed from 2 $\mu$g of total RNA with oligodeoxythymidine as initiation primer. PCR amplification of a 5′ fragment of the pig $\alpha$Gal coding region was done with the following primers: 5′-AGG AAG AGT GGT TCT GTC-3′, corresponding to nucleotides 12 through 30 of pig $\alpha$1,3GT isoform 1, and 5′-GTT ATG GTC ACG ACC TCT-3′, corresponding to nucleotides 324 through 306 of pig $\alpha$1,3GT isoform 1, as previously described elsewhere.[16,17] PCR reaction mix consisted of 100 pmol primers, 10 mmol/L-deoxyribonucleoside triphosphates, 3-mmol/L magnesium chloride, 10-mmol/L Tris hydrochloride buffer, 50-mmol/L potassium chloride, 10% glycerol, and 2 U *Taq* polymerase. Conditions for PCR reaction were as follows: 94°C denaturing temperature for 30 seconds, 54°C hybridization for 30 seconds, and 72°C elongation temperature. PCR was performed for 35 cycles. PCR reactions were resolved on precast 4% agarose gels and stained with ethidium bromide. Images were recorded with Polaroid 667 film (Polaroid Corporation, Cambridge, Mass).

## Western Blot

Porcine aortic endothelial cells, porcine valve endothelial cells, and human vein endothelial cell monolayers were washed with phosphate-buffered saline solution containing 5-mmol/L EDTA, scraped from the flasks in phosphate-buffered saline solution and EDTA, and centrifuged at 900*g* for 5 minutes at 4°C in a swinging bucket centrifuge. Cell pellets were then lysed in 1 volume of lysis

buffer (50-mmol/L Tris [pH 7.2] containing 2% Triton X-100 [The Dow Chemical Company, Midland, Mich], 5-mmol/L EDTA, and protease inhibitors [pepstatin A, leupeptin, bestatin, aprotinin, and E-64]) for 1 hour on ice. After sedimentation of nuclei and debris at 10,000*g* for 30 minutes, supernatants were collected and stored at −80°C until use. Protein concentration was determined by the bicinchoninic acid method, and 20 μg of total protein per lane was analyzed by immunoblotting. Aliquots of endothelial cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on an 8% gel under reducing conditions according to the method of Laemmli.[18] A second gel was run as a control to ascertain equal loading and transfer, which was confirmed by staining with Coomassie blue. Proteins were then transferred to a 0.45-μmol/L polyvinylidene difluoride membrane at 125 V for 2 hours and stained with biotinylated IB4 lectin, according to minor modifications of a previously described method.[19] In brief, biotinylated IB4 lectin was used at a 1:100 concentration (diluted in phosphate-buffered saline solution) for 1 hour at room temperature after blocking overnight with 0.2% casein and Tris-buffered saline Tween (0.05%) solution at 4° C. The membrane was then washed three times for 5 minutes each in phosphate-buffered saline solution. The Vectastain Elite kit was then used according to directions to develop the immunoblot. It was critical to eliminate Tween from the wash and incubation buffers, because the IB4 lectin displays no signal in the presence of stringent wash conditions (data not shown). Control blots were run in the absence of lectin and stained with 3,3′-diaminobenzidine with virtually no background signal. As an additional control, blots included a lane of human vein endothelial cell total protein extract, which is known not to express αGal. All experiments were performed in triplicate, with a single representative blot being shown.

### Flow Cytometry

Cells were allowed to grow to near confluence and then harvested with trypsin, which was neutralized in phosphate-buffered saline solution with 1% bovine serum albumin, washed twice with phosphate buffered-saline solution, and resuspended in phosphate-buffered saline solution with 1% bovine serum albumin. Flow cytometric analysis was performed according to minor modifications of previously published protocols[20] by incubating 100 μL ($2 \times 10^5$ cells) of porcine aortic or valve endothelial cells or human vein endothelial cells with 100 μL of fluorescein isothiocyanate-conjugated IB4 lectin (20 μg/mL) in phosphate-buffered saline solution with 0.5-mmol/L calcium chloride for 30 minutes on ice. After double washing with phosphate-buffered saline with 0.5-mmol/L calcium chloride, the cells were resuspended in phosphate-buffered saline solution and analyzed on an EPICS-XL flow cytometer (Coulter Corporation, Hialeah, Fla) at 488 nm. Human vein endothelial cells, which are known not to express αGal, served as a negative control for the IB4 lectin. All experiments were performed twice with one representative scan shown.

## Results

### Establishment of Porcine Valve Endothelial Cell Culture

Because there are few standard descriptions and protocols for the culture of primary porcine valve endothelial cells,[21,22] initial experiments were established to determine the proper growth parameters. Cells grew rapidly when placed on a 2% bovine gelatin substrate but not on standard cell culture dishes (data not shown). Cells had typical cobblestone morphologic character and exhibited contact inhibition in the presence of 20% fetal calf serum (data not shown).

### Immunohistochemical Studies

Cells were initially characterized by staining with the IB4 lectin to determine αGal reactivity. Whereas porcine aortic endothelial cells (Figure 1, *A*) stained intensely with IB4 lectin, porcine valve endothelial cells (Figure 1, *B*) showed minimal immunoreactivity for the IB4 lectin. Porcine myocardium (Figure 1, *C*) served as both positive and negative tissue controls for the lectin. Porcine myocardium stained intensely in the capillaries and arterioles for αGal, whereas cardiomyocytes were completely unstained and larger epicardial vessels showed minimal reactivity. Cells were routinely photographed at 450× magnification and tissue sections were routinely photographed at 250× magnification.

To confirm that these were endothelial cells, we stained them with the RCA lectin (βGal), which has been previously shown to be a mammalian endothelial cell marker.[23] Both aortic (Figure 1, *D*) and valve (Figure 1, *E*) endothelial cells stained positively for RCA, confirming their endothelial origin. As a control, myocardium was stained, as shown in Figure 1, *F,* with the RCA lectin. Endothelial cells, both microvascular and large epicardial vessels, appeared to stain intensely with this lectin, which is consistent with previously published reports.[24]

As further confirmation, immunohistochemical testing was performed for smooth muscle cell α-actin. Both aortic (Figure 1, *G*) and valve (Figure 1, *H*) endothelial cells had minimal reactivity toward smooth muscle cell α-actin. As a staining control, porcine myocardium was stained with the same antibody (Figure 1, *I*). There was appropriate staining in the smooth muscle layers of both arteries and veins.

In summary, valvular endothelial cells in culture exhibited the same IB4 staining patterns (RCA positive, αGal negative) in vitro as in vivo with respect to endothelial cell phenotype.

### Western Blot

Whole-cell homogenates were obtained for analysis of IB4 by immunoblotting. Twenty micrograms of total protein from three different experiments was loaded for both porcine valve and aortic endothelial cells. Human vein endothelial cells were included as a control, and a molecular weight marker was used. There was an intensely positive αGal band in aortic endothelial cells at 135 kd (Figure 2); this band was lacking in porcine valve endothelial cells and human vein endothelial cells. A less intense band was evident at 125 kd in valve endothelial cells.

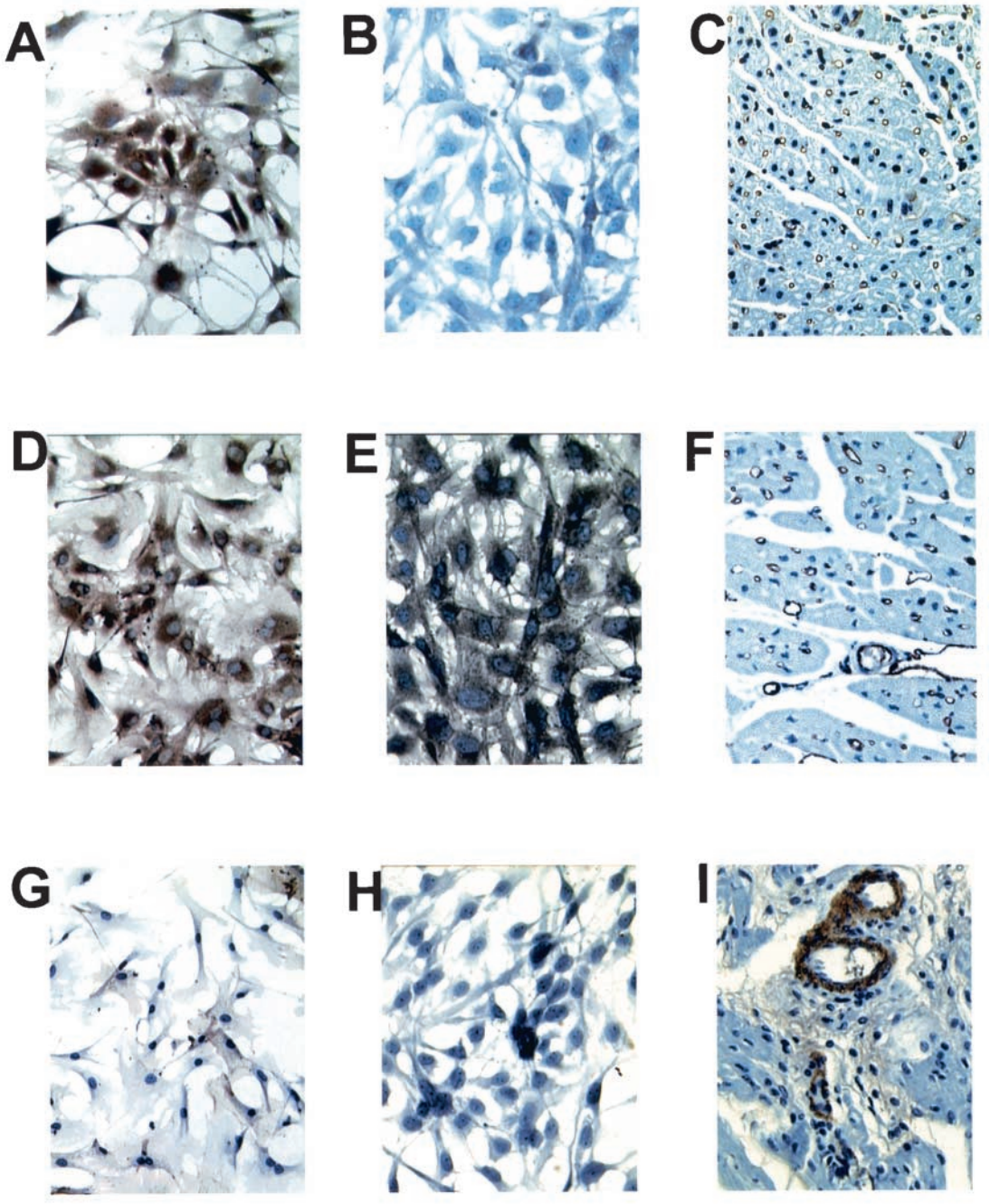


**Figure 1. Characterization by immunohistochemical staining of various cell types. A, Aortic endothelial cells stained with IB4 lectin to detect $\alpha$Gal staining. B, Valve endothelial cells stained with IB4 lectin to detect $\alpha$Gal staining. C, Porcine myocardium stained with IB4 lectin to detect $\alpha$Gal staining. D, Aortic endothelial cells immunohistochemically stained with $\beta$Gal, documented mammalian endothelial cell marker. E, Valve endothelial cells immunohistochemically stained with $\beta$Gal, documented mammalian endothelial cell marker. F, Porcine myocardium immunohistochemically stained with $\beta$Gal, documented mammalian endothelial cell marker. G, Aortic endothelial cells immunohistochemically stained with antibody directed against smooth muscle cell $\alpha$-actin. H, Valve endothelial cells immunohistochemically stained with antibody directed against smooth muscle cell $\alpha$-actin. I, Porcine myocardium immunohistochemically stained with antibody directed against smooth muscle cell $\alpha$-actin. All cells photographed at 450× magnification. Tissues photographed at 250× magnification.**

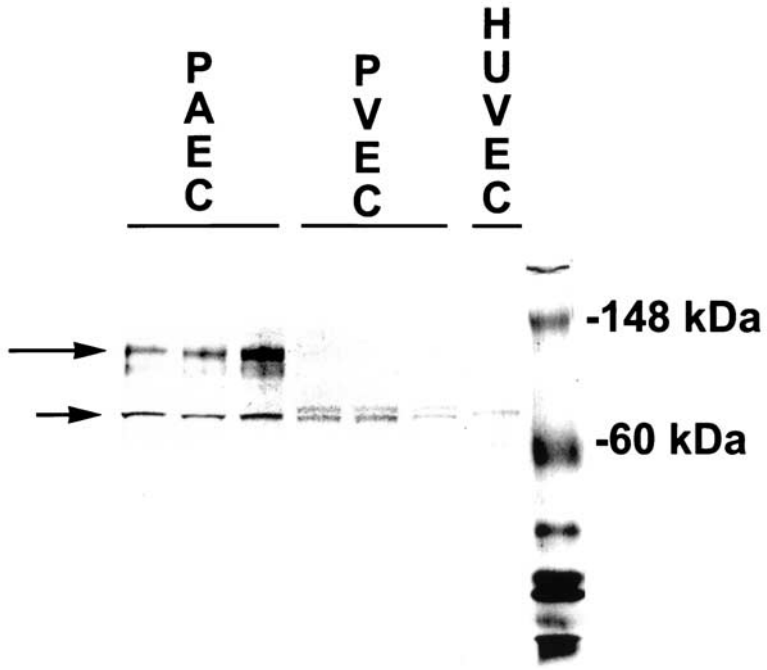


**Figure 2. Western blot of porcine aortic endothelial cells *(PAEC)* versus porcine valvular endothelial cells *(PVEC)* in 6% polyacrylamide gel. In lanes *1* through *3* (from left) there is 20 μg of porcine aortic endothelial cell protein per lane. In lanes *4* through *6* there is 20 μg of porcine valvular endothelial cell protein per lane. In lane 7 there is human vein endothelial cell *(HUVEC)* protein as control. In lane 8 there is protein standard. *Large arrow* points to 135-kd band, whereas *small arrow* corresponds to 115-kd band.**



### Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from porcine aortic and valve endothelial cells by the acid-guanidinium-thiocyanate method. Two micrograms of total RNA was then reverse transcribed into complementary DNA. Two hundred nanograms of complementary DNA, corresponding to a 10th of the reaction, was then amplified with primers for human β-actin and porcine αGal. Three amplified products were visualized for the αGal gene in both porcine aortic and valve endothelial cells (Figure 3). These corresponded to previously published sizes and distribution patterns.[17] Although reverse transcriptase-PCR is not an absolutely quantifiable method but rather a relative one, it was obvious that both samples contained similar amounts of amplified product, in the same sizes, with similar amounts of β-actin, a constitutive gene that was coamplified. Sequencing revealed that base pairs were 286, 313, and 349 nucleotides in length. Films were scanned and intensity was measured. In contrast to our findings with immunohistochemical and Western blot analyses, there were no significant differences in band intensity between transcripts from aortic and valve endothelial cell messenger RNA.

### Flow Cytometry With the IB4 Lectin

To determine whether there was any difference in the surface profile of IB4 reactivity in intact cells, flow cytometry was performed with fluorescein isothiocyanate-conjugated IB4. Porcine aortic and valve endothelial cells and human vein endothelial cells were harvested and mixed with fluorescein isothiocyanate-conjugated IB4 and allowed to react for 30 minutes on ice. Cells were then analyzed at 488 nm by flow cytometry, and the curves are shown superimposed. In agreement with our messenger RNA analysis, both aortic and valve endothelial cells were almost identical in fluorescence intensity, in contrast to human vein endothelial cells (which do not express αGal and served as a negative control; Figure 4).

## Discussion

To clarify the immunogenicity of porcine valve endothelium, we investigated the mechanisms underlying the expression of the αGal epitope on porcine valve endothelial cells. αGal is the major xenoreactive epitope in the pig and as such is the antigen responsible for hyperacute rejection of porcine xenografts. We have previously shown that there is no immunohistochemically detectable αGal on valve endothelial cells, although there is intense αGal staining in capillaries.[8,25] As such, valve endothelium may represent immunologically privileged tissue, suggesting a potential role for a fresh cardiac valve prosthesis.

Results of this investigations shed new light on the apparent lack of αGal epitopes on cardiac valve endothe-

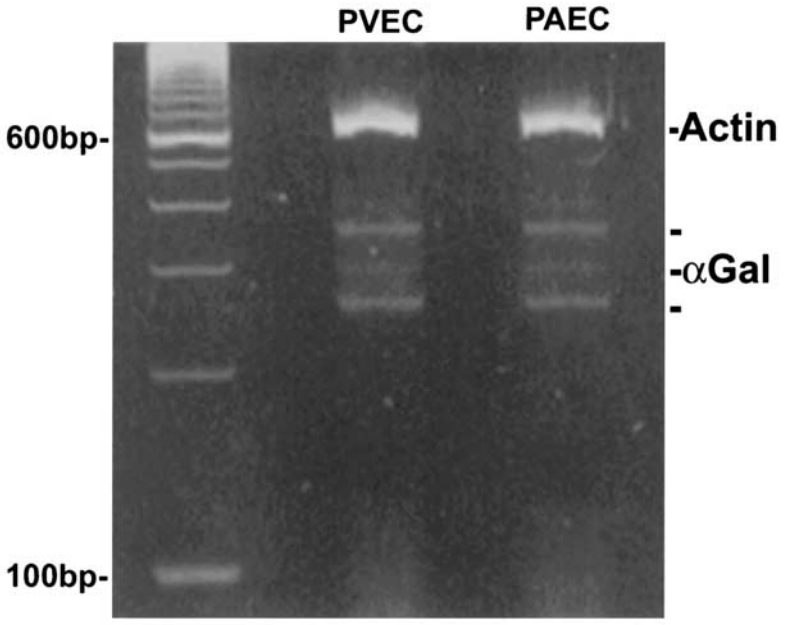


**Figure 3. Reverse transcriptase-PCR of porcine aortic endothelial cells *(PAEC)* versus porcine valve endothelial cells *(PVEC)*. Cells were grown to confluence, RNA was extracted and reverse transcribed, and transcripts were amplified with primers for αGal and β-actin. In lane *1* there is 100-bp ladder with intense band at 600 bp. In lanes *2* and *3* there are porcine valve endothelial cell and porcine aortic endothelial cell extracts. β-Actin (661 bp) and αGal (3 transcripts at sizes 286, 313, and 349 bp) demonstrate equal intensity bands.**

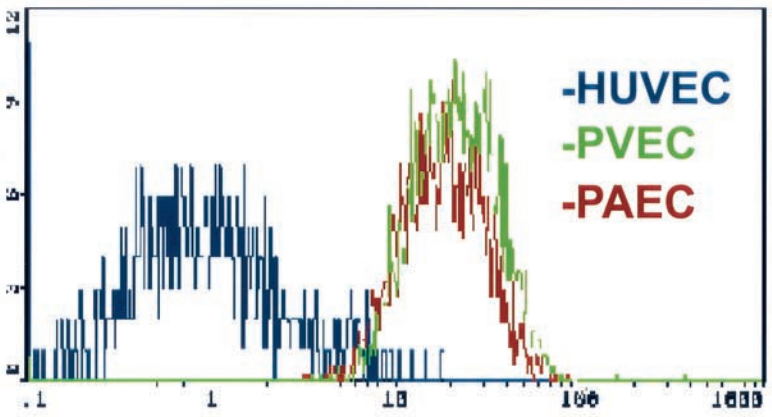


**Figure 4. Flow cytometry of human vein endothelial cells *(HUVEC)*, porcine valve endothelial cells *(PVEC)*, and porcine aortic endothelial cells *(PAEC)*. To assess surface immunoreactivity of endothelial cells, fluorescein isothiocyanate-conjugated labeled IB4 was incubated for 1 hour.**

lium. Whereas immunohistochemical and Western blot analyses documented decreased αGal reactivity in valve endothelial cells, flow cytometry and reverse transcriptase-PCR demonstrated nearly equivalent surface epitope and transcript profiles for αGal, implying that immunostaining with the IB4 lectin may be ineffective in identifying relevant αGal epitopes.

First, we established a method for the primary cultures of valve endothelial cells. We documented that cultured valve endothelial cells appeared to have similar phenotypic expression to that on valve leaflets. For example, cultured valve endothelial cells exhibited typical cobblestone morphologic type and were RCA lectin positive, confirming their endothelial lineage.[23] Furthermore, in contrast to porcine aortic endothelial cells, valve endothelial cells expressed almost no αGal according to IB4 immunostaining. This finding is similar to our

previous reports,[8,9] in which we documented that there was no IB4 detectable staining on the endothelium of porcine cardiac valves before or after xenotransplantation into primates. We were also able to confirm by Western blot analyses that valve endothelial cells expressed less IB4-detectable αGal than did aortic endothelial cells. This is in good agreement with our previous histologic results.

Reverse transcriptase-PCR demonstrated αGal gene expression on both aortic and valvular endothelial cells, also demonstrating equivalent amounts of transcript in the two cell types. The α1,3galactosyltransferase gene is alternatively spliced from a single genomic locus that synthesizes four proteins with variations in the length of the Golgi-spanning stem region. These four isoproteins have all been shown to have α1,3galactosyltransferase activity, with four possible transcripts of 250, 286, 313, and 349 bp.[26] Here we demonstrated three transcripts, which is consistent with previously published reports.[17] This is the first report to our knowledge of the differential display of these transcripts in porcine valve endothelial cells. When compared with a constitutive gene, such as the β-actin gene, there are equal amounts of all three transcripts.

Finally, flow cytometry with the IB4 lectin revealed identical surface profiles in aortic and valve endothelial cells, which were distinct from human vein endothelial cells. This is consistent with the reverse transcriptase-PCR activity of these cells. Apparently the IB4 lectin did not have sufficient sensitivity in the harsh environment of histochemical and Western washes to detect low levels of αGal epitope expressed on the endothelial cell surface. In the more gentle conditions of flow cytometry required for lectin-αGal interactions, it is possible that there is a tighter IB4-αGal interaction.

Most studies investigating the expression of αGal have used the IB4 lectin from *B (Griffonia) simplicifolia* for immunohistochemical[23,27,28] or fluorescence-activated cell sorter analyses.[20] This lectin is from a family of tetravalent lectins that bind αGal, with the IB4 lectin completely specific for αGal. Although its specificity is high, the sensitivity of the IB4 lectin may be insufficient for the detection of small numbers of the αGal epitope. There is a low binding affinity of each monomeric interaction of the IB4 lectin with αGal. Because of this, it has been postulated that if the αGal epitopes are not in close enough proximity to enable a IB4-αGal interaction with more than one molecule of the lectin (which has four combining sites), then the lectin may be removed during overly stringent washes.[29,30] This may explain why, despite messenger RNA and fluorescence-activated cell sorter analyses demonstrating transcript and IB4-detectable αGal epitopes, respectively, there is minimal staining by immunohistochemical means. Also, posttranscriptional modifications of glycoproteins or differences in αGal presentation have been noted to affect the relative abundance of immunodetectable αGal.[31]

Other tissue beds that have been thought to be immunologically privileged have been shown to have low-level expression of αGal epitopes. Indeed, it has been demonstrated that despite a low expression of αGal epitopes in bovine and porcine cartilage, there is a marked (20- to 100-fold) increase in anti-αGal activity when cartilage is placed into suprapatellar pouches in cynomolgus monkeys.[32,33] This emphasizes the need to remove all αGal epitopes, because even a very low expression may be sufficient to propagate a chronic rejection cascade.

This study confirms that porcine valve endothelium expresses αGal epitopes in vitro, although these epitopes are not detectable by immunohistochemical means. Furthermore, equivalent amounts of α1,3galactosyltransferase messenger RNA isoforms are present in valve and aortic endothelial cells, suggesting that any differences may be due to posttranscriptional modifications or differences in antigen presentation. Unless all αGal epitopes are removed from the endothelium, such as by targeted gene disruption, our results suggest that fresh porcine valves are ultimately likely to trigger delayed xenograft rejection.

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